

## Contribution of putrescine degradation to proline accumulation in soybean leaves under salinity

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### Abstract

Proline accumulation was studied in the leaves of *Glycine max* (L.) Merr. subjected to salt stress in the presence of aminoguanidine (AG, a specific inhibitor of diamine oxidase, DAO) and exogenous putrescine (Put). Both DAO activity and proline content were increased while endogenous Put content was decreased in soybean leaves under 50 to 150 mM NaCl. There was a negative correlation between proline accumulation and endogenous Put content. The addition of AG during NaCl stress inhibited DAO activity, caused Put accumulation and a 15 to 20 % decrease in proline content. Application of 1 mM Put to NaCl solution markedly increased proline content. The promotive effect of Put application could be alleviated by the treatment with Put plus AG. Moreover an application of AG had no effect on proline accumulation in soybean seedlings grown under normal condition. These results indicate that the quantitative contribution of Put degradation to proline formation is 15 to 20 %.

*Additional key words:* *Glycine max*, NaCl-induced stress, polyamine degradation.

Accumulation of proline and changes in endogenous contents of free polyamines (PAs) are considered to play an important role in plant responses to salt stress (Bouchereau *et al.* 1999, Lefevre *et al.* 2001, Gaspar *et al.* 2002). To date, a number of studies mentioned the correlation between PA and proline contents in salt stressed plants due to a close relationship between PAs and proline metabolism. It is well-known that both PAs and proline possess a common precursor-glutamate. Glutamate can be directly converted to proline through glutamate- $\Delta^1$ -pyrroline-5-carboxylate pathway or indirectly to polyamines *via* its acetylation in ornithine and arginine (Santa-Cruz *et al.* 1999, Gaspar *et al.* 2000, Zhao *et al.* 2001, Tonon *et al.* 2004). Santa-Cruz *et al.* (1999) reported that a decline in Put content was parallel to proline accumulation in salt tolerant tomato when NaCl concentration increased up to 200 mM. However, Sanchez *et al.* (2005) found that PAs did not always parallel proline accumulation in *Lotus glaber* under long-term salt stress. Furthermore, Tonon *et al.* (2004) reported that proline content highly paralleled Put content under non-lethal NaCl concentrations while different changes in proline

and Put occurred under lethal NaCl concentration.

Diamine oxidase (DAO) activity is promoted by high salt stress (Smith 1985, Aziz *et al.* 1998, Su *et al.* 2007). PAs, such as Put and Spd, degraded *via* DAO (EC: 1.4.3.6) and polyamine oxidase (PAO, EC: 1.5.3.11), can contribute to proline accumulation through  $\gamma$ -amino-butyric acid production (Bouchereau *et al.* 1999, Gaspar *et al.* 2000). In some species such as tomato and soybean submitted to salt stress, PA degradation was promoted (Aziz *et al.* 1998, Su *et al.* 2007) and proline content significantly increased (Tonon *et al.* 2004, Sotiropoulos 2007). To our knowledge, not much information is available on a quantitative contribution of PA degradation induced by salt stress to proline accumulation.

The enzyme involving in PA degradation in soybean is DAO, not PAO whose activity has still not been detected (Rea *et al.* 1998). Though Spd and Spm are also substrates for DAO activity, only the products of Put degradation can be converted to proline (Bouchereau *et al.* 1999). Moreover, our previous studies found that DAO activity in soybean leaves could be promoted by salt stress (Su *et al.* 2007). So soybean is a good material for studying the

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*Abbreviations:* AG - aminoguanidine; DAO - diamine oxidase; PAs - polyamines; PAO - polyamine oxidase; Put - putrescine; Spd - spermidine; Spm - spermine.

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interrelation between Put and proline. The objective of the present research was to reveal a quantitative contribution of Put degradation by salt stress to proline accumulation.

Soybean [*Glycine max* (L.) Merr.] cv. Suixie-1 seeds were sterilized with 0.5 % HgCl<sub>2</sub> for 5 min, soaked for 6-h in distilled water after being washed five times, and then germinated at 25 ± 1 °C for 24-h in moist filter paper in plastic boxes. The uniformly germinated seeds were selected, planted in silica boxes supplemented with half-strength Hoagland solution, and cultivated at 14-h photoperiod, irradiance of a 340 μmol m<sup>-2</sup> s<sup>-1</sup> and day/night temperature of 28/22 ± 1 °C in the growth chamber. After two weeks, seedlings were gathered, and then cultivated in 1/2 Hoagland solution containing 1) 0, 50, 100, 150 mM NaCl; 2) 0, 0.5, 1.0 mM AG; 3) 0, 0.5, 1.0 mM AG + 100 mM NaCl; 4) 0, 1.0 mM Put, 1.0 mM Put + 100 mM NaCl, 1.0 mM Put + 1.0 mM AG +100 mM NaCl. All solutions were adjusted to pH 5.8 and renewed every two days. All samples were cultivated under conditions mentioned above. After one week of treatments, leaf mass, proline and endogenous PA contents and DAO activity were determined.

Using a UV-visible spectrophotometer (UV-1700, Shimadzu, Tokyo, Japan), DAO activity in soybean leaves was estimated by the method described previously (Su *et al.* 2005). Briefly, leaf tissues (0.5 g) were ground at 4 °C in 1.6 cm<sup>3</sup> 0.1 M sodium phosphate buffer (pH 6.5) containing 5 % (m/v) polyvinylpyrrolidone (PVP). Homogenate was centrifuged at 10 000 g for 20 min at 4 °C. Reaction mixture (3.0 cm<sup>3</sup>) contained 2.5 cm<sup>3</sup> 0.1 M sodium phosphate buffer (pH 6.5), 0.1 cm<sup>3</sup> crude enzyme extracts, 0.1 cm<sup>3</sup> peroxidase (250 U cm<sup>-3</sup>) and 0.2 cm<sup>3</sup> 4-aminoantipyrine/N,N-dimethylaniline solution. The reaction was initiated by the addition of 0.1 cm<sup>3</sup> 20 mM Put. A change in absorbance at 555 nm of 0.01 was regarded as unit of the enzyme activity. Proline content was determined according to the method described by Troll and Lindsley (1955). Leaf samples (0.5 g) were homogenized in 4 cm<sup>3</sup> 3 % 5-sulfosalicylic acid. After bathing in boiling water and cooling to room temperature, the homogenate was centrifuged (2800 g, 10 min). The supernatant (0.2 cm<sup>3</sup>) was mixed with 2 cm<sup>3</sup> of ice acetic acid and 2 cm<sup>3</sup> of ninhydrin reagent. The mixture was boiled for 30 min and the reaction stopped in an ice-bath. The chromophore obtained was extracted with 4 cm<sup>3</sup> toluene by vortexing for 30 s. Absorbance was measured

at 520 nm with the UV-1700 spectrophotometer. For determination of free PAs, high performance liquid chromatography (HPLC) as described by Flores and Galston (1982) was used. The samples were extracted with 4 cm<sup>3</sup> 5 % (v/v) HClO<sub>4</sub>. After bathing in an ice water and centrifuging (15 000 g, 20 min) at 4 °C, 0.5 cm<sup>3</sup> of the supernatants was mixed with 1 cm<sup>3</sup> 2 M NaOH. After the addition of 10 mm<sup>3</sup> benzoylchloride, vortexing for 20 s, and incubating for 20 min at 37 °C, 2 cm<sup>3</sup> ether was added. After centrifugation (1500 g, 5 min), 1 cm<sup>3</sup> of the ether phase was collected, evaporated to a dry state, and redissolved in 100 mm<sup>3</sup> methanol (HPLC grade). Standards were treated in a similar way, with up to 50 nmol of Put, Spd and Spm, respectively, in the reaction mixture. Benzoyl-polyamines (10 mm<sup>3</sup>) was analyzed using Waters 1515 HPLC System (Denver, USA) consisting of an isocratic pump equipped with a reverse phase C<sub>18</sub> column (Nova-pak, 150×3.9 mm, particle size 4 μm). Methanol/acetonitrile/H<sub>2</sub>O (48/2/50) (v/v/v) was used as an isocratic eluting solvent at a flow rate of 1 cm<sup>3</sup> min<sup>-1</sup>. The means of three or four separate experiments of different treatments were used to calculate Student *t*-test.

Leaf fresh and dry masses gradually decreased as NaCl concentrations increased. Under 50, 100 and 150 mM NaCl stress, leaf fresh mass was decreased by 3.9, 22.2 and 45.6 %, respectively. Dry mass was reduced by 3.2, 12.5 and 32.9 % under similar conditions. In order to maintain osmotic equilibrium under salt stress, plants usually accumulate some compatible solutes with low molecular mass such as proline (Ashraf and Harris 2004, Tripathi *et al.* 2007, Sotiropoulos 2007). In our research, an increase of 7-, 13- and 21-fold in proline content was observed relative to the control after 6 d of 50, 100 and 150 mM NaCl stress, respectively (Table 1). To evaluate polyamine catabolism under salinity, endogenous polyamine contents and DAO activity were determined. The results showed that both Put and Spd contents decreased with increasing NaCl concentrations, while Spm content increased by 2.7 to 2.8 times under similar conditions. DAO activity is thought to be related to endogenous polyamine contents. It was found that DAO activity was increased to 1.4-, 2.0- and 3.6-fold under 50, 100 and 150 mM NaCl. The changes in DAO activity were in agreement with those in Put and Spd contents (Table 1). There was a negative correlation between proline accumulation and endogenous Put content. In plant

Table 1. Effects of different NaCl concentrations on leaf mass, contents of proline, Put, Spd and Spm, and DAO activity in soybean seedlings. Means of three or four separate experiments ± SE. Different letters show means significantly different at *P* < 0.05.

NaCl [mM]	Leaf fresh mass [mg plant <sup>-1</sup> ]	Leaf dry mass [mg plant <sup>-1</sup> ]	Proline [μmol g <sup>-1</sup> (d.m.)]	Put [nmol g <sup>-1</sup> (d.m.)]	Spd [nmol g <sup>-1</sup> (d.m.)]	Spm [nmol g <sup>-1</sup> (d.m.)]	DAO activity [U g <sup>-1</sup> (d.m.) min <sup>-1</sup> ]
0	779.01 ± 46.02a	124.34 ± 4.04a	6.82 ± 1.68d	371.3 ± 21.4a	545.0 ± 18.1a	101.3 ± 8.0b	7.0 ± 0.4d
50	748.56 ± 57.89a	120.31 ± 3.01a	48.26 ± 4.92c	237.6 ± 9.0b	372.2 ± 13.2b	284.5 ± 3.1a	9.5 ± 0.5c
100	606.35 ± 38.46b	108.80 ± 3.04b	86.68 ± 7.01b	167.4 ± 6.1c	200.6 ± 13.8c	286.6 ± 21.7a	14.0 ± 0.8b
150	424.21 ± 27.61c	83.42 ± 3.64c	146.97 ± 11.89a	71.0 ± 6.4d	66.5 ± 6.1d	272.0 ± 6.6a	25.1 ± 1.2a

Table 2. Changes in proline and endogenous Put contents and DAO activity after treatments with different AG concentrations or exogenous Put in salt stressed soybean leaves. Means of three or four separate experiments  $\pm$  SE. Different letters show means significantly different at  $P < 0.05$ .

Treatments	Proline [ $\mu\text{mol g}^{-1}(\text{d.m.})$ ]	Put [ $\text{nmol g}^{-1}(\text{d.m.})$ ]	DAO [ $\text{U g}^{-1}(\text{d.m.}) \text{min}^{-1}$ ]
Control	6.61 $\pm$ 1.37 d	338.8 $\pm$ 18.4 c	6.54 $\pm$ 0.74 b
0.5 mM AG	6.20 $\pm$ 1.89 d	455.5 $\pm$ 27.6 a	0.86 $\pm$ 0.05 c
1.0 mM AG	6.77 $\pm$ 1.43 d	489.9 $\pm$ 20.8 a	0.51 $\pm$ 0.04 c
100 mM NaCl	89.51 $\pm$ 3.79 b	167.8 $\pm$ 11.2 d	14.5 $\pm$ 1.49 a
100 mM NaCl + 0.5 mM AG	76.10 $\pm$ 1.55 c	373.5 $\pm$ 25.8 b	1.03 $\pm$ 0.13 c
100 mM NaCl + 1.0 mM AG	71.00 $\pm$ 1.09 c	407.1 $\pm$ 13.3 b	0.86 $\pm$ 0.09 c
1.0 mM Put	7.58 $\pm$ 0.74 d	-	-
100 mM NaCl + 1.0 mM Put	103.51 $\pm$ 2.30 a	-	-
100 mM NaCl + 1.0 mM Put + 1.0 mM AG	78.27 $\pm$ 2.87 c	-	-

responses to salt stress, this correlation between PA and proline was also found in other species, such as tomato (Aziz *et al.* 1998, Santa-Cruz *et al.* 1999), *Fraxinus angustifolia* callus (Tonon *et al.* 2004) and *Lotus glaber* (Sanchez *et al.* 2005). One of the explanations for this change is that both PAs and proline compete for glutamate as a common precursor (Santa-Cruz *et al.* 1999, Tonon *et al.* 2004). A competitive correlation occurs in the synthetic pathways of PAs and proline, especially under severe salt stress (Gaspar *et al.* 2000, Zhao *et al.* 2001), thus showing an increase in proline and a decrease in PAs. Besides, a decrease in Put and Spd contents in salt stressed plants can be assigned to a reduction in arginine decarboxylase and S-adenosyl-L-methionine decarboxylase activities (Maiale *et al.* 2004), and to conversion of Put and Spd to Spm. Maiale *et al.* (2004) found that salt stress decreased Spd synthase activity in salt tolerant rice, and that salt stress plus cyclohexylamine, an inhibitor of Spd synthase, did not reduce Put content, probably due to Spd synthase inhibition. As shown in our research (Table 1), Spm accumulated and its accumulation seemed to be a general trend in plants under salt stress (Santa-Cruz *et al.* 1997, Maiale *et al.* 2004, Sanchez *et al.* 2005). The finding that *A. thaliana* Spm synthase gene AtSPMS was induced by salt shock provides a molecular basis for this conversion (Urano *et al.* 2003). However, due to increased DAO activity in salt stressed soybean leaves (Table 1), this negative correlation between proline and PA contents might be related to the conversion of PA degradation product to proline. Smith (1985) found that DAO and PAO activities in oat seedlings were promoted by salt stress. Aziz *et al.* (1998) reported that aminoguanidine, a specific DAO inhibitor, could strongly inhibit proline accumulation in salt stressed tomato leaf discs.

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When 100 mM NaCl was coupled with 0.5 or 1.0 mM AG, proline content in salt stressed soybean leaves decreased (Table 2). The decreasing rate was 15.0 to 20.6 %, suggesting the contribution of PA degradation to proline accumulation. Interestingly, AG treatment had no effect on proline accumulation in the leaves of soybean grown under normal conditions although it caused an increase in Put content (34.4 to 44.6 % and 122.3 to 145.6 % increase occurred both in non-salt stressed and in salt stressed soybean leaves). In order to further confirm the contribution of PA degradation to proline accumulation, 1.0 mM Put was added. While 1.0 mM Put did not increase proline content in the control, together with 100 mM NaCl caused a 15.7 % increase in proline. Surprisingly, the promotive effect of exogenous Put could be alleviated addition of 1.0 mM AG. The results provide further evidence for the 15 to 20 % contribution of PA degradation products to proline accumulation in salt stressed soybean leaves. AG treatment powerfully inhibited the DAO activity, but only decreased proline content by about 15.7 % (Table 2), suggesting that the Put degradation products are probably one of the sources leading to proline accumulation under salt stress. In fact, proline accumulation in plant responses to salt stress appears to be related with *de novo* synthesis and decrease of its degradation (Hien *et al.* 2003, Parida and Das 2005). Moreover, lower proline utilization in protein synthesis and increased proteolysis may also contribute to proline accumulation (Hien *et al.* 2003, Parida and Das 2005).

Using AG, a DAO specific inhibitor, and exogenously applied Put, this study confirmed that a 15 to 20 % of proline accumulated in soybean leaves under NaCl stress can come from PA (Put) degradation products.

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This voluminous book contains one introductory essay dealing with life work of an excellent scientist plus 29 reviews dealing with various topics of actual plant research. They were prepared by 68 well-known scientists from all over the world.

The introductory paper describes life and discoveries of E.E. Conn, an American plant biochemist whose name is connected with cyanogenic plants, those that contain hydrogen cyanide in a bound form releasing it when the plant tissue is macerated. The cyanogenic glycosides are usually linked to D-glucose. In contrast to other reviews of the volume, nine photographs of scientists and only a selection of cited papers are presented.

The following papers can be divided into about ten groups according to their main topic. The questions of signalling, sensory photoreceptors, and genetic regulatory

network are one of them. The introductory review deals with nitric oxide signalling which modulates the activity of plant proteins and mobilizes calcium, and compares the mechanisms in plants and animals. Sensory photoreceptors in algae include proteins, cryptochromes, phototropins, phytochromes, and rhodopsins; the review shows how they regulate algae development, photosynthesis, orientation in nature, and control their circadian clock. Plant phytochromes and interacting proteins are another important subject, not only limited by the well-known red/far-red mechanism. The role of metabolism of trehalose and its products in signalling and coordination of metabolism with plant development are stressed in another review. Ubiquitin and cullin ligases are related to hormonal signalling, plant development, and responses to environmental factors.